Comm.
Dr. Andervont
Dr. Gardner
Dr. Huebner

CARCINOGENESIS

#904

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N.Y. 10022

MAR 8 1973

RECEIVED

Application For Research Grant

Date: February 22, 1973

1. Name of Investigator(s): (include Title and Degrees)

David Apirion, Ph.D.
Associate Professor of Microbiology

2. Institution & Address:

Washington University
Lindell & Skinker
St. Louis, Missouri 63130

3. Short Title of Project:

Alterations in nuclei associated with malignancy

4. Proposed Starting Date: 1 July 1973

5. Anticipated Duration of this Specific Study: 30 June 1976

6. Brief Descripton of Objectives or Specific Aims:

1) The development of a two dimensional gel electrophoresis system amenable to autoradiography for the analysis of chromosomal proteins.

2) Comparison of total chromosomal proteins in transformed and non-transformed lines. This will be tested in material isolated from cromatin, from total metaphase chromosomes and from single chromosomes.

3) Comparison of acetylation phosphorylation and other modifications of chromosomal proteins in transformed and nontransformed lines. This will be tested in material isolated from chromatin, total metaphase chromosomes and from single chromosomes.

4) Isolation of differentiation variants and analysis of their chromosomal proteins.

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7. Give a Brief Statement of your Working Hypothesis: The behaviour of a malignant cell, for instance unrestricted growth, is due to changes in the response of its genome to environmental (cellular) stimuli. The capacity of the genome to respond to these stimuli depends to a large extent on nuclear proteins.

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8. *Details of Experimental Design and Procedures: (Attach Separate Pages)*

(See pages 5-11.)

9. Physical Facilities Available (Where Other than Administering Organization Indicate Geographical Location)

General facilities at our disposal include controlled temperature rooms (30°, 37°), media room, autoclaves, cold rooms (4°, -18°), and a -70° refrigerator. Preparative and analytical ultracentrifuges with a number of rotors. Amino acid analyzer. A scintillation and a dry counter for radioisotopes. High voltage electrophoretic apparatus. Spacious lab area and office. A number of water baths and incubators, small centrifuges, and various other facilities necessary in a modern biochemical laboratory. We have excellent animal quarters, and fairly adequate facilities for tissue culture work. Spacious lab space is available.

10. Additional Requirements:

Most of our present tissue culture facilities are concentrated in a central laboratory which is next to my lab and serves all the department. Therefore the initial work can be started even without addition of any new equipment; however, for the continuation of the project facilities for tissue culture work would have to be introduced into my laboratory.

11. Biographical sketches of all principal and professional personnel (append)

(See pages 12 & 13.)

12. List of publications: (Five most recent as pertinent) (append)

(See page 14.)

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13. Budget (1st year)

A. Salaries (Personnel by names)	% time	Amount
Professional		
David Apirion		
Ruth Kaplan		
Technical		
Edward Watson		
Vera Johnson		
Ernestine Love		
Fringe Benefits (Social Security)		
	Sub-Total	
B. Consumable Supplies (list by categories)		
Liquid Nitrogen		400
Tissue Culture Medium		2,200
Chemical Substrates and Enzymes		1,450
Radioisotopes		1,650
Glassware and Plasticware		1,100
Animals and animal supplies		600
	Sub-Total	7,400
C. Other Expenses (itemize)		
National Scientific meeting for two persons		700
Publications costs (page charges, illustration, reprints, typing)		300
Books and Journals		100
Equipment Maintenance Contracts		500
	Sub-Total	1,600
D. Permanent Equipment (itemize)		
Water Bath for Spinners with Magnetic Rotors		1,000
Laminar Flow Hood		1,500
Inverted Microscope		1,200
Electrophoresis Units (2)		800
Power Supply Units (2)		1,600
		6100
E. Overhead (15% of A+B+C)		5,791
	Total	$50,496

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Estimated Future Requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Overhead	Total
Year 2	31,381	7,750	1,800	3,500	6,140	$50,571
Year 3	33,264	8,000	2,000	2,400	6,490	$52,154

It is understood that the applicant and institutional officers in applying for a grant have read and found acceptable the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Signature David Apirion
Director of Project (314) 367-6400 Ext. 3451 Telephone

Signature Joe F. Evans
Business Officer of the Institution Joe F. Evans
(314) 863-0100, Ext. 4570 Telephone

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Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

	Title of Project	Source	Amount	Duration
Current				
	Genetic and Structural Studies of E. coli Ribosomes	National Science Foundation	$ 32,346	5/15/72-5/14/73
	A Biological Approach to Controlled Synthesis of Desired Proteins	National Science Foundation	16,135	11/1/72-4/30/74
Pending				
	Studies with Neuroblastoma Lines	American Cancer Society	109,978	7/1/73-6/30/75
	Chromosomal Proteins in Normal and Malignant Cells	Damon Runyon Memorial Fund for Cancer Research Inc.	89,446	7/1/73-6/30/75
	The Fate of RNA in Malignant and Normal Cells	National Institutes of Health	151,542	9/1/73-8/31/76

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Introduction. A malignant cell can be viewed as a cell which has lost part of its regulatory capacity, a cell which ceases to respond to various stimuli as does the precursor cell.

Parts of the genome of a mammalian cell seem to be permanently blocked, while some other parts seem to be capable of responding to stimuli in the cellular environment. Cells from different tissues in the organism or in tissue culture can respond to similar effector molecules, such as cAMP. However, their responses vary: fibroblast cells and neuroblastoma cells both respond to cAMP but very different proteins are produced and different processes apparently occur in both cells during these responses. That part of the genome which responds to the effector must be determined by something in the cell. Since in all likelihood genomes of different tissues, in the same organism, are by and large identical, the differences among nuclei from different tissues could be due to chromosomal proteins.

Since the genome of a malignant cell ceases to behave as does a genome of the parental nontransformed cell it is quite likely that a change occurred in the chromosomal proteins.

There is a growing body of evidence linking changes in the genome with specific cancers; see for instance references 1 - 3. However, these changes do not seem to be too specific, i.e. it is not clear whether the genome is altered or whether the change could be related to alterations in chromosomal proteins. A much more interesting observation is that the synthesis of nuclear acidic proteins is increased after SV40 infection (4). In order to analyze such situations (transformed versus nontransformed cells) we wish first to develop proper techniques for qualitative and quantitative analysis of chromosomal proteins and to use these techniques on specific cell lines where the analysis could be most informative.

Materials. We plan to work exclusively with mouse cells. In order to be able to isolate specific chromosomes (see below) we wish to use a specific mouse line that contains only one metacentric chromosome. Such a strain was isolated by crossing Mus musculus to Mus poschiavinus and back crossing the hybrid to the first parent (5). (Mus poschiavinus contains seven metacentric chromosomes.) The single metacentric chromosome in the strain isolated from the back cross is distinct and it replaces two acrocentric musculus chromosomes. In all likelihood it should be feasible to separate this chromosome from all others. Other mouse lines are available in which by reciprocal translocations between two acrocentric chromosomes one larger chromosome and one smaller chromosome have been produced. In such a line it might be possible to separate individually two chromosomes, the large and the small, from all other chromosomes. Separation will have to be carried out on cells grown in tissue culture arrested in the metaphase stage.

Isolation of chromatin, chromosomes and their analysis. Chromatin and chromosomes would be isolated from cells transformed by polyoma virus or other means and nontransformed cells, and from cells during the process of cell transformation (chromatin). (Techniques for such isolations are available and some of the references are mentioned above.) As chromosomes are separated

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the cells would be labeled first (long term) with ^{14}C-amino acids in order to label the chromosomal proteins; thus even very small quantities of material could be analyzed by autoradiography.

Prior to the isolation of the chromatin and chromosomes we shall develop two dimensional polyacrylamide gel electrophoresis for the analysis of the chromosomal proteins. At present this technique developed by Kaltschmidt and Wittmann (6), is probably the most sensitive technique available for identification of proteins in a mixture. Moreover ribosomal proteins and chromosomal proteins both share common characteristics, especially with respect to solubility, and therefore it sould be quite easy to adapt the existing techniques to the analysis of both the histones and the acidic proteins. For further details about the analysis, see below.

The qualitative and quantitative pattern of the chromosomal proteins in the chromatin and chromosomes isolated from transformed and nontransformed cells, would be compared. This analysis would be augmented by proper in vivo labelling of the proteins with radioisotopes. Thus we should be able to determine whether or not there is a unique pattern to chromosomal proteins during cell infection with a virus, during the establishment of transformation and after the process is finished and the condition is maintained. Similar analysis would be carried out on cells transformed by other means, e.g. 3T3 mouse cells and cells derived from them which have lost contact inhibition.

Acetylation and phosphorylation of chromosomal proteins. Chromosomal proteins, and specifically acidic proteins, are known to undergo acetylation and phosphorylation which seems to be relevant to either the cell cycle or to regulation of gene function. These parameters would be measured on total chromosomes, chromatin and isolated chromosomes. This would be done during regular growth and after infecting cells with the virus. In a different approach chromosomal isolated chromosomes and the isolated proteins would be used as substrates to measure acceptance of phosphate by protein phospho kinase mediated by cAMP.

Such comparisons as described in the last two sections coupled with knowledge about gene activity of a specific chromosome (see below) would lead to meaningful correlations between gene activity or gene restriction and transformation. We should be able to determine whether there is any indication that the programming written into the cells during differentiation is changed during and after transformation.

Variants of Differentiation. A very interesting situation that ties together a viral infection and differentiation is the cloned line of murine proerythroblastoid cells. Such a line can be transformed by infecting it with Friend leukemia virus (7, 8). Such transformed cells can be made to differentiate by addition of dimethylsulfoxide (Me_2SO) to the culture medium (9).

In this system the comparison of chromosomal proteins, their phosphorylation and acetylation, can be made among the three stages - the uninfected cells, the infected transformed cells and the differentiated cells. Since recently it was shown that during this differentiation process a specific messenger is produced (globin mRNA; 10) it should be feasible to find out what specific changes in the nucleus, in chromosomal proteins, were caused during this viral

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the cells would be labeled first (long term) with ^{14}C-amino acids in order dependent differentiation process.

This phenomenon mentioned above suggests that it should be possible to study nuclear changes during transitions from one differentiated stage to another. Another case that we shall study is the transition in neuroblastoma cells from undifferentiated to differentiated cells, which can be caused by small effector molecules such as cAMP (11).

It should be possible to isolate from existing cell lines variants that can produce proteins which are not usually produced. For instance, a cell line developed from a fibroblast such as a 3T3 line does not produce globin or liver proteins such as serum albumin. However it has been shown by Petterson and Weiss (12) that hybridization of a mouse fibroblast line with a rat hepatoma line gave a hybrid which started producing mouse serum proteins. Therefore we should select lines that produce new products, with and without transforming the line with a virus. First, we shall find out whether the frequency with which such new variants, referred to herein as differentiation mutants, can be increased after transformation. If this is the case it would suggest that changes in the genome which led to transformation and malignancy are relevant to changes in the genome which lead to differentiation. A number of agents, such as alkylating agents, nitrous acid, acridines, acridine half mustards, etc., would be used to induce such variants, and from finding out which of them is effective some insight could be gained toward what causes this process.

There are a number of possible ways to score or select for such variants, (differentiation mutants) of which we mention here only a few.

1) Selection for the production of an enzyme that can be stained for histochemically,
2) Selection for heme production (stain with benzidin), or autoradiography with iron,
3) Selection for serum albumin producing cells.

1) The enzyme acetyl choline esterase: Nirenberg and his coworkers (13) showed that it is possible to stain neuroblastoma colonies for this enzyme. Therefore 3T3 cells would be mutagenized or treated in various ways, e.g., with DMSO, and colonies would be produced and stained for production of the enzyme acetyl choline esterase. Since this is a visible reaction it should be feasible to screen a large number of colonies in this assay. Should such variants be found, the process would be repeated under conditions where each colony is grown in duplicate, so that further analysis of any suspect colony (which probably would be killed by the staining procedure) could be continued. Growing of large number of colonies in an ordered fashion is possible in multiwelled plates (see ref. 14). We have ascertained that we can grow mouse L cells in such units.

2) Detection of heme producing lines: this could be accomplished either by staining with benzidin, or by autoradiography with iron (15), using techniques similar to those developed by Milcarek and Weiss (16) for the isolation of DNAseless mutants of E. coli. We are at present isolating RNAseless mutants of E. coli by this technique, and are satisfied with it. I am confident that it is a very adequate technique for the isolation of variants

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that might show a change in incorporation of a specific radioisotope.

3) Isolation of serum albumin producing cells from a 3T3 fibroblast line: For this purpose an antibody against purified mouse serum albumin would be prepared, (excellent facilities are available in our department for this purpose) and colonies arising from treated (mutagenized) cells would be treated with the antibody in the manner with which Sharff and colleagues (17) treat colonies to find out whether or not they produce antibodies.

There is the possibility that the serum protein is synthesized but not secreted, and is thus not detected by the antibody. If after screening a reasonable number of colonies (about 10,000) none producing serum albumin that should be detected, treated (mutagenized) cells would be inoculated into the small wells. The beauty of these multiwell plates is that they are treated in or as test tubes and therefore we shall open the cells grown in them and then treat them with the antibody in agar. Thus a precipitate could be formed and observed. Again, if this fails, the antibody can be labeled by an iodine radioisotope and an antigen antibody complex can be screened for by autoradiographic techniques.

I would like to emphasize that the three characteristics described here are only examples, and many other phenotypes can be screened for. Therefore I have no doubt that the isolation of "differentiation variants" is feasible and the analysis of the chromosomal proteins and their chemical modification in such lines should be very illuminating and very critical for the understanding of cell differentiation. I would like also to point out that in these screenings or selections, we shall be looking for positive traits and therefore even if the colonies we analyze are mixed clones arising from some modified and some unmodified cells, we should be able to discover these clones, since they should score as positive or partial positive in the tests.

Isolation and analysis of specific chromosomes. There are two main aims in the analysis of unique chromosomes; 1) concentration on a unique chromosome some of the active genes of which might be known and determining specifically whether there are any quantitative or qualitative changes or modifications in its proteins, during or after the process of cell transformation, or in differentiation variants. 2) Location of similar parameters about a chromosome to which the virus actually attached. While it probably would be desirable to know the latter, knowing the former would be helpful to determine whether specific changes occur in the nucleus (chromosomes) after cell transformation. Although the isolation of a specific chromosome and its analysis may be achieved it cannot, of course, be predicted that the virus would attach specifically to the chromosome that we plan to isolate. However, this might be achieved by using the mouse man or mouse-Chinese hamster cell-cell hybridization.

In a hybrid cell line of mouse and man (18), using a human viral transformed line, the human chromosomes would carry the SV40 viral genome. In such a line it should be possible to isolate clones that lost most of the human chromosomes. It should be also possible to hybridize a mouse transformed cell with a human nontransformed cell or Chinese hamster cell and to isolate hybrids that carry small numbers of the mouse chromosomes. A hybrid line would be searched for that carries just a single human or mouse chromosome (the mouse chromosome should be relatively easy to identify) and which still carries the virus (this can be verified by analysis

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of specific viral antigens). While the hybrids would usually lose preferentially the human chromosomes, it should be possible by irradiation of the mouse cells prior to the hybridization, to cause preferential loss of mouse chromosomes (19). Perhaps among such specific hybrids there will be one from which the mouse chromosome could be separated from all the others. This should allow us to analyze the chromosomal proteins of a chromosome that carries a viral genome.

It is clear that this project has a number of experimental approaches, and success in any of them would make our efforts worthwhile and very rewarding. The independent parts of the project are the following: a) Development of two dimensional gel electrophoresis for chromosomal proteins, b) Autoradiography for acetylation and phosphorylation of chromosomal proteins; this depends on a but can be done independently by one dimension gel electrophoresis. c) Isolation of a single chromosome from a mouse, d) Isolation of differentiation variants, e) Isolation of a chromosome carrying a viral genome.

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13. Minna, J., Glazer, D., and Nirenberg, M. (1972). Genetic dissection of neural properties using somatic cell hybrids. Nature New Biology 235: 225-231.
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19. Pontecorvo, G. (1971). Induction of directional chromosome elimination in somatic cell hybrids. Nature 230:367-369.

Work and experience relevant to this project

For my master and Ph.D. thesis I worked on genetics of higher organisms (20-25) which gives me a basic familiarity with genetic problems of eukaryotic cells. During my studies for the Ph.D. I developed a two-way selection technique (see ref. 2). Similar principles are used now in the selection of resistant mutants in tissue culture which become simultaneously auxotrophic. Also I had the benefit of working with Professor G. Pontecorvo (1961-63) at the time in which he initiated somatic cell genetics in tissue culture. During my studies for the Master's degree I prepared and taught cytogenetic laboratories which should help me now with the chromosomal aspects of the project. Also this year I taught a graduate course on eukaryotic chromosomes, which has further orientated me towards this research problem. My experience with ribosomal proteins (25-27) would, I believe, be most useful since the techniques we use to analyze ribosomal proteins are most appropriate to the analysis of chromosomal proteins as in both cases one is dealing with complex organelles which are composed of nucleic acids and proteins.

For analysis of ribosomal proteins we use a variety of polyacrylamide gels, round gels, one dimension slab gels and two dimension gels (the first dimension of electrophoresis is done in a round gel and the second is in a slab gel). Also recently we introduced thin (1.5mm) one dimensional slab gels which are adequate for autoradiography, since they can be easily dried. We have already made some preliminary trials with nuclear proteins isolated from chromatin of mouse liver cells and mouse L-cells using the thin layer one dimensional slab gels. It is clear from the results that before long we should have at our disposal very powerful tools for the analysis of chromosomal proteins. We chose to develop a technique amenable to autoradiography for two reasons: 1) we anticipate that most of the work on proteins isolated from chromosomes would have to be carried out on very small amounts of material where identification would have to depend

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on radioisotopes; 2) an important part of the analysis of chromosomal proteins would involve modifications that are taking place in them during a variety of cellular events related to transformation and malignancy. Such physiological modifications can be detected by proper radioisotope labeling.

In order to learn cell hybridization techniques I spent during 1971 a number of weeks in Dr. F. Ruddle's laboratory (Yale University) who is one of the leaders in this field. In all the specific areas which are outside my expertise there are a number of people in the Medical School who are very competent in these areas and who have promised help and counsel if necessary.

PERTINENT REFERENCES

20. Apirion, D., and Zohary, D. (1961). Chlorophyll lethal in natural populations of the orchard grass (Dactylis glomerata L.) A case of polymorphism in plants. Genetics, 46: 393-399.

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22. Apirion, D. (1963). Formal and physiological genetics of ascospore colour mutants in Aspergillus nidulans. Genet. Res. 4: 276-283.

23. Apirion, D. (1965). The two-way selection of mutants and revertants in respect of acetate utilization and resistance to fluoo-acetate in Aspergillus nidulans. Genet. Res. 317-329.

24. Apirion, D. (1966). Recessive mutants at unlinked loci which complement in diploids but not in heterokaryons of Aspergillus nidulans. Genetics, 53:935-941.

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CURRICULUM VITAE

NAME: David Apirion SOC. SEC. NO. R

DATE & PLACE OF BIRTH: REDACTED

CITIZENSHIP: R

MARITAL STATUS: REDACTED

EDUCATION:

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Glasgow University, Glasgow, Scotland	Ph.D.	1960-63	Biology Genetics

FIELD OF SPECIALIZATION: Molecular Biology and cellular organization.

PRESENT POSITION:

Associate Professor, Washington University School of Medicine, Department of Microbiology, St. Louis, Missouri — 1970 to present

PROFESSIONAL EXPERIENCE AND APPOINTMENTS:

Research and teaching assistant, Hebrew University, Jerusalem, Israel	1959-60
Research Student, Glasgow University, Glasgow, Scotland	1960-62
Assistant Lecturer, Glasgow University, Glasgow, Scotland	1962-63
Research Fellow, Harvard University, Cambridge, Massachusetts	1963-65
Assistant Professor, Washington University, Department of Microbiology, St. Louis, Missouri	1965-70

HONORS:

The Tuvia Kushnir Prize	1960
The Alexander Milman Prize	1961
The Sir Maurice Bloch Award	1962

SOCIETY MEMBERSHIPS:

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PUBLICATIONS

David Apirion

M.S. Thesis (1960) Distribution of albino genes in populations of Dactylis glomerate, in Israel.

Ph.D. Thesis (1963) Studies on the selection of mutants in Aspergillus nidulans.

1. Apirion, D., and Zohary, D. Chlorophyll lethal in natural populations of the orchard grass (Dactylis glomerate L.) A case of balanced polymorphism in plants. Genetics 46: 393-399 (1961).

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3. Apirion, D. Formal and physiological genetics of ascospore colour in Aspergillus nidulans. Genetics Res. 4: 276-283 (1963).

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12. List of five pertinent publications (reprints enclosed)

1. Deusser, E., Stoffler, G., Wittmann, H. G., and Apirion, D. Ribosomal proteins: XVI. Altered S4 proteins in Escherichia coli revertants from streptomycin dependence to independence. Molec. Gen. Genetics, 109: 298-302 (1970).

2. Stoffler, G., Deusser, E., Wittmann, H. G., and Apirion, D. Ribosomal proteins. XIX. Altered S5 ribosomal protein in an Escherichia coli revertant from streptomycin dependence to independence. Molec. Gen. Genetics 111:334-341 (1971).

3. Simon, M., and Apirion, D. A messenger RNA from the lactose operon of Escherichia coli that can not direct the production of functional β-galactosidase in absence of exogenous adenosine 3'-5'-cyclic monophosphate. Genetics, 7: 1-18 (1972).

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5. Meyhack, B., Meyhack, I., and Apirion, D. Colicin E3: A unique endoribonuclease. Proc. Natl. Acad. Sci. U.S.A. 70: 156-160 (1973).

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